REMARKS

The Office Action dated January 17, 2002 has been received and reviewed. Claims 1 through 20 are pending in the present application. Claims 5 and 13 have been withdrawn from consideration. Claims 1 through 4, 6 through12 and 14 through 20 stand rejected. The application is to be amended as previously set forth. All amendments are made without prejudice or disclaimer. Reconsideration is respectfully requested.

1. **Priority**

Applicants claim priority from an International Patent Application PCT/EP99/07800 filed 06 October 1999 designating the United States of America, which itself claims priority from European Patent Application EP 98203529.7 filed 20 October 1998. Applicants hereby acknowledge the Examiner's remarks regarding receipt of the required foreign priority document and acknowledge that unless such document is filed as provided in and required by 35 U.S.C. § 119 (a)-(d) and 37 C.F.R. § 1.55, enjoyment of the benefit of foreign priority to the EPO document will be denied. A copy of the priority document has been requested and will be supplied upon receipt by the undersigned.

2. **Election/Restrictions**

Applicants hereby affirm the election without traverse made during a telephone conversation between the Examiner and Mr. Allen Turner on December 12, 2001 to prosecute the invention of Group I, claims 1-20 and the species election encompassing *Lactococcocus sp*, Crohn's disease, immunosuppressive drugs and IL-10. The pending claims have been amended herein to reflect this election. Applicants further acknowledge that claims 5 and 13, as well as the non-elected species, have been withdrawn from further consideration by the Examiner according to 37 C.F.R. § 1.142(b).

3. **Claim Objections**

Claims 9 and 17 have been objected to for reciting a gramatically improper phrase. Specifically, the Examiner has objected to the phrase "co-administration of the at least . . .". Claims 9 and 17 have been amended as suggested by the Examiner to instead recite the phrase "co-administration of at least . . .". Applicants respectfully submit that the objection to claims 9 and 17 has been obviated and, accordingly, request withdrawal thereof.

4. **Claim Rejections under 35 U.S.C. § 112, first paragraph**

Claims 1 through 4, 6 through12 and 14 through 20 have been rejected under 35 U.S.C. § 112, first paragraph. Specifically, the Examiner has stated that while the specification is enabled for 1) a method of treating colitis in a mammal, comprising administering a medicament comprising an amount of a genetically modified *Lactococcus lactis* expressing IL-10 to a mammal with colitis by injecting the recombinant *Lactococcus lactis* into the peritoneum of the mammal, wherein the administration results in an increase in colon length and reduced epithelial damage and infiltration of lymphocytes in the mammal with colitis; and 2) a method as recited in claim 1, wherein the medicament is administered in combination with at least one additional anti-colitis drug, the specification assertedly is not enabled "for other claimed embodiments embraced by the breadth of the claims." (Office Action, pages 8 and 9) Applicants respectfully traverse the rejection for the following reasons.

First, applicants respectfully traverse the Examiner's assertion that the present invention is "directed to a method of treating a mammal by methods of ex vivo gene therapy . . .". (Office Action, page 9) *Ex vivo* gene therapy involves extracting cells from the subject to be treated, genetically altering the extracted cells and transplanting the genetically altered cells back into the subject. *See, Nature*, Vol. 389, pp. 239-242, 1997 (hereinafter the "Verma reference") at p. 240. The present invention does not relate to such *ex vivo* gene therapy, but rather relates to recombinant bacteria introduced into the gastrointestinal tract of the subject to be treated which produce, *in situ*, a cytokine which aids in the prevention or healing of inflammatory bowel disease. Accordingly, it

respectfully is submitted that the cited references relating to the state of the art regarding vectors and methods useful for gene therapy are not relevant to the present invention. Specifically, it is submitted that the Anderson reference (*i.e.*, *Nature*, Vol. 392, pp. 25-30, April 1998) and the Verma reference are not relevant to that which is recited in the claims herein.

Further, applicants respectfully submit that the present invention is not related to DNA vaccination. Accordingly, the state of the art regarding DNA vaccinations which is taught by the McCluskie reference (*i.e.*, *Molecular Medicine*, 5, pp. 287-300, 1999) also is not relevant to the claims of the present application.

As claimed, the present invention relates to the use of a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain for treating inflammatory bowel disease. Particular Gram-positive bacterial strains for which the present invention may be used include *Bacillus subtilis*, *Streptococcus gordonii*, *Staphylococcus xylosus* and *Lactobacillus species* such as *L. bulgaricus*, *L. salivarius*, *L. casei*, *L. helveticus*, *L. delbrueckii* and *L. plantarum*. (*See*, specification, para. **[0038]**.) All of the bacterial strains listed above are non-invasive strains. The present invention is not intended to suggest using harmful bacterial strands such as *Staphylococcus areus*, *Enterococcus spp* and *Streptococcus pneumoniae* and the Examiner's point in this regard is well-taken. (*See*, Office Action, p. 12) Independent claim 1 and dependent claims 3 and 11 have been amended herein to clarify that only non-invasive Gram-positive bacterial strands are intended to be encompassed by the present invention. Applicants respectfully submit that the state of the art does provide sufficient guidance for one skilled in the art to make and/or use a representative number of non-invasive Gram-positive bacteria, such as those listed above, in the method of the present invention.

The surprising results obtained by the present invention relate to introducing any molecule known to affect inflammatory bowel disease into the gut of the subject to be treated so that the molecule can be locally produced by a bacterium that acts as a carrier and as a production unit. Thus, the present invention teaches a method for introducing ***any cytokine*** into the gut. Applicants respectfully submit that one skilled in the art would be able, without undue experimentation, to

extrapolate from the *L. lactis* examples provided by the present invention to other non-invasive Gram-positive bacteria in the method of the present invention. The specification provides ample guidance to a person skilled in the art regarding how to treat a subject with inflammatory bowel disease by administering a suitable Gram-positive bacterium (*e.g.*, a non-invasive Gram-positive bacterium) as a carrier/production item for any cytokine known to affect the prevention or cure of inflammatory bowel disease.

Further, it respectfully is submitted that the mouse model disclosed by the present invention is a generally recognized model for treatment of inflammatory bowel disease in any subject. In support of this contention, an article authored by the inventors of the present invention and published in the renowned journal *Science* (*Science* 2000: 1352-1355) submitted herewith as Appendix D. It is stated in the abstract of the article that "[t]his approach may lead to better methods for cost-effective and long-term management of IBD *in humans*" (emphasis added). A statement such as this undoubtedly would not be published in a renowned peer-reviewed journal like *Science* if the extrapolation from mice to other mammals was unacceptable to those of skill in the art.

Applicants respectfully submit that the culmination of the above remarks establishes that a person skilled in the art is given sufficient guidance in the present application regarding how to treat any subject with inflammatory bowel disease by administering a suitable non-invasive Gram-positive bacterium as a carrier/production item for any cytokine known to affect prevention or cure of the disease. Accordingly, reconsideration and withdrawal of the 35 U.S.C. § 112, first paragraph, rejection of claims 1 through 4, 6 through 12 and 14 through 18 thus respectfully is requested. Claims 19 and 20 have been cancelled by way of the present amendment and thus the rejection as to these claims has been rendered moot.

5. **Claim rejections under 35 U.S.C. §102**

Claims 1 through 4, 6, 10 through 12, 14 and 18 through 20 have been rejected under 35 U.S.C. § 102(b) as being anticipated by WO 96/11277 to Tagliabue et al. (hereinafter the "Tagliabue

reference") as evidenced by Herfarth et al. (hereinafter the "Herfarth reference"). Applicants respectfully traverse the rejection.

As amended herein, independent claim 1 recites a method of treating inflammatory bowel disease in a subject, the method comprising administering to the subject a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain, *wherein administering the medicament results in reduction of inflammation by at least 50% and/or prevention of onset of inflammation.* The emphasis indicates a limitation added by virtue of the present amendment, support for which may be found in paragraph **[0041]** of the as-filed specification.

Applicants respectfully submit that the Tagliabue reference neither expressly nor inherently suggests a method of treating inflammatory bowel disease having each of the elements recited in independent claim 1. Specifically, the Tagliabue reference does not suggest, either expressly or inherently, that administration of a cytokine-producing non-invasive Gram-positive bacterial strain results in a reduction of inflammation of at least 50% during inflammatory bowel disease and/or in prevention of the onset of inflammatory bowel disease as is specifically recited by the present invention.

The present invention solves the problem of administering an engineered bacterium which can efficiently prevent and/or cure (by at least 50%) inflammation during inflammatory bowel disease. Faced with this problem, one of ordinary skill in the art would have to undergo an undue amount of experimentation in light of the Tagliabue reference to solve the problem. Moreover, one skilled in the art certainly could not predict that inflammatory bowel disease could be efficiently prevented or cured at all consulting only the Tagliabue reference and the state of the art generally prior to the present invention.

The Tagliabue reference recognizes the problem of surpassing a gastric environment in case bacteria are administered orally and states that spore-forming bacteria *should* reach the intestinal mucosa (*see*, p. 12, lines 13-23, emphasis added). However, the Tagliabue reference fails to offer any examples demonstrating how (and how many) bacteria should be orally administered, if any

bacteria will pass the gastric environment, or if bacteria which do pass the stomach will still be able to produce *de novo* a therapeutically effective amount of protein. Furthermore, the Tagliabue reference shows that administration of IL-1ra-producing *Bacillus subtilis* bacteria directly into the colon via a catheter reduces mortality due to endotoxic shock by 30% to 40% depending upon the dose of LPS (*see*, pp. 41-42, example 6 and table 4). However, the Tagliabue reference does not disclose any studies concerning oral administration, how to treat inflammatory bowel disease, or what to expect if inflammatory bowel disease were to be treated. The Tagliabue reference merely mentions (p. 17, lines 22-31) that inflammatory bowel disease is a type of disease which is treatable, among eleven other types of diseases, by administration of the pharmaceutical compositions described in that reference. Applicant respectfully submits that this alone does not permit one skilled in the art to draw the conclusion that the onset of inflammatory bowel disease can be prevented or that inflammation due to the disease can be reduced by at least 50%.

Thus, a person of ordinary skill in the art could certainly not predict if treatment with engineered bacteria would, with a reasonable expectation of success, efficiently prevent or cure (by at least 50%) inflammatory bowel disease. Thus, based upon the undue amount of experimentation that would be required based only upon the Tagliabue reference and the state of the art generally prior to the present invention, and there being no reasonable expectation of success, it is clear that the present invention is novel over the prior art of record. Applicants further submit that the teachings regarding exogenous IL-10 as applied to colitis assertedly disclosed in the Herfarth reference do not, when combined with the Tagliabue reference, result in the latter reference anticipating the present invention for the reasons discussed above.

Independent claim 1 is believed to be in condition for allowance and applicants, accordingly, respectfully request withdrawal of the 35 U.S.C. § 102(b) rejection thereof. Each of claims 2 through 4, 6, 10 through 12, 14 and 18 depend, either directly or indirectly, from independent claim 1 and thus are believed to be in condition for allowance for at least the above-stated reasons. As such, applicants respectfully request withdrawal of the 35 U.S.C. § 102(b) rejection of these claims

as well. Claims 19 and 20 have been cancelled herein and thus the rejection of these claims has been rendered moot. Favorable action respectfully is requested.

6. **Claim rejections under 35 U.S.C. §103**

Claims 1, 2, 7, 8, 9, 15, 16 and 17 have been rejected under 35 U.S.C. § 103(a) as being unpatentable over the Tagliabue reference taken in combination with either *The Gastroenterologist*, Vol. 3, 1995, pp. 141-152 (hereinafter the "Korelitz reference") or U.S. Patent 6,262,119 to Ferrante et al. (hereinafter the "Ferrante reference"). As a *prima facie* case of obviousness under 35 U.S.C. § 103 cannot be established based upon the Taglibue, Korelitz or Ferrante references, or any combination thereof, applicants respectfully traverse the rejection.

In order to establish a *prima facie* case of obviousness under 35 U.S.C. § 103, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference(s) or to combine the reference teachings. "The mere fact that reference can be combined or modified does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination [or modification]." *In re Mills*, 916 F.2d 680, 1 USPQ2d 1430 (Fed. Cir. 1990). *See also, ACS Hospital Systems, Inc. v. Monteffore Hospital*, 732 F.2d 1572, 1577, 221 USPQ 929, 933 (Fed. Cir. 1984) (stating that "[o]bviousness cannot be established by combining the teachings of the prior art to produce the claimed invention, absent some teaching, suggestion or incentive supporting the combination."). Second, there must be a reasonable expectation of success. Third, the cited prior art reference must teach or suggest all of the claim limitations. Furthermore, the suggestion to make the claimed combination and the reasonable expectation of success both must be found in the prior art and must not be based upon applicants' disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

In light of the foregoing, applicants respectfully submit that the Examiner has failed to establish a *prima facie* case of the obviousness of the present invention based upon the Tagliabue reference, the Korelitz reference, the Ferrante reference, or any combination thereof.

As previously stated, amended claim 1 of the present invention recites a method of treating inflammatory bowel disease in a subject, the method comprising administering a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain to the subject, wherein administering the medicament results in reduction of inflammation by at least 50% and/or prevention of onset of inflammation.

First, the cited prior art references fail to teach or suggest all of the limitations of independent claim 1. As discussed previously, the Tagliabue reference neither teaches nor suggests a method of treating inflammatory bowel disease wherein administering the medicament results in reduction of inflammation by at least 50% and prevention of onset of the inflammation. Applicants respectfully submit that the Korelitz reference and the Ferrante reference similarly lack such teaching or suggestion. Furthermore, none of the references is relied upon for such teaching by the Examiner.

Further, no suggestion or motivation exists, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine the reference teachings to achieve a method of treating inflammatory bowel disease wherein administering the medicament results in reduction of inflammation by at least 50% and prevention of onset of inflammation. The mere fact that the references can be combined and/or modified does not render the resultant combination obvious absent some suggestion concerning the desirability of the combination and/or modification. Further, applicants respectfully submit that, even if the cited references were combined, an undue amount of experimentation would be necessary to achieve a method as recited in the present invention and that one skilled in the art would not reasonably expect success upon such modification or combination. (*See supra*)

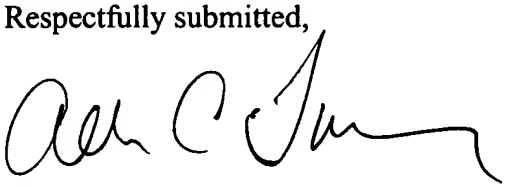
In view of the foregoing, it respectfully is submitted that a *prima facie* case of obviousness of independent claim 1, as amended herein, cannot be supported based upon the cited references. Accordingly, claim 1 is believed to be in condition for allowance and applicants respectfully request withdrawal of the 35 U.S.C. § 103(a) rejection of this claim. Each of claims 2, 7 through 9 and 15 through 17 depend, either directly or indirectly from independent claim 1 and thus are believed to

be in condition for allowance for at least the above-cited reasons. Favorable action respectfully is requested.

CONCLUSION

In view of the present amendment and the above remarks, claims 1 through 4, 6 through 12 and 14 through 18 are believed to be in condition for allowance and an early notice thereof respectfully is solicited. Should the Examiner determine that additional issues remain which might be resolved by a telephone conference, he respectfully is invited to contact applicants' attorney at the address or telephone number given herein.

Respectfully submitted,



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Enclosures: Appendix A
Appendix B
Appendix C
Appendix D

APPENDIX A

(MARKED-UP VERSION OF CLAIMS TO SHOW CHANGES MADE)
(Serial No. 09/838,718)

Version of Claims with Markings to Show Changes Made

1. (Amended) A method of treating inflammatory bowel disease in a subject, said method comprising:

administering a medicament comprising an amount of a cytokine-producing non-invasive Gram-positive bacterial strain ~~or a cytokine antagonist-producing non-invasive Gram-positive bacterial strain~~ to said subject, wherein the administration of said medicament results in one or more of reduction of inflammation by at least 50% and prevention of onset of said inflammation.

2. (Amended) The method according to claim 1 wherein the cytokine ~~or cytokine antagonist~~ is ~~selected from the group consisting of~~ IL-10~~, a soluble TNF receptor or another TNF antagonist, an IL-12 antagonist, an Interferon-γ antagonist, an IL-1 antagonist, a virus-coded cytokine analogue, and EBV BCRF1~~.

3. (Amended) The method according to claim 1 wherein the non-invasive Gram-positive bacterial strain is a *Lactococcus* species.

6. (Amended) The method according to claim 1 wherein the bowel disease is ~~selected from the group consisting of chronic colitis,~~ Crohn's disease ~~and ulcerative colitis~~.

8. (Amended) The method according to claim 7 wherein the at least one therapeutic agent ~~is selected from the group consisting of corticosteroids, sulphasalazine, derivatives of sulphasalazine,~~ includes at least one immunosuppressive ~~drugs, cyclosporin A, mercaptopurine,~~

~~azathioprine, and another cytokine~~ drug.

9. (Amended) The method according to claim 7 wherein the co-administration of ~~the~~ at least one additional therapeutic agent is sequential or simultaneous.

10. (Amended) The method according to claim 1 wherein the medicament is delivered through *in situ* synthesis by recombinant ~~*L.*~~ *Lactococcus lactis*.

11. (Amended) The method according to claim 2 wherein the non-invasive Gram-positive bacterial strain is a *Lactococcus* species.

14. (Amended) The method according to claim 2 wherein the bowel disease is ~~selected from the group consisting of chronic colitis,~~ Crohn's disease ~~and ulcerative colitis~~.

16. (Amended) The method according to claim 15 wherein the at least one therapeutic agent ~~is selected from the group consisting of corticosteroids, sulphasalazine, derivatives of sulphasalazine,~~ includes at least one immunosuppressive ~~drugs, cyclosporin A, mercaptopurine, azathioprine, and another cytokine~~ drug.

17. (Amended) The method according to claim 15 wherein the co-administration of ~~the~~ at least one additional therapeutic agent is sequential or simultaneous.

18. (Amended) The method according to claim 2 wherein the medicament is delivered through *in situ* synthesis by recombinant ~~*L.*~~ *Lactococcus lactis*.

19. (Cancelled)

20. (Cancelled)

APPENDIX C

(VERSION OF SUBSTITUTE SPECIFICATION EXCLUDING CLAIMS WITH MARKINGS TO SHOW CHANGES MADE)
(Serial No. 09/838,718)

PATENT
Attorney Docket 4779

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APPLICATION FOR LETTERS PATENT

for

USE OF A CYTOKINE-PRODUCING *LACTOCOCCUS* STRAIN TO TREAT COLITIS

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USE OF A CYTOKINE-PRODUCING *LACTOCOCCUS* STRAIN TO TREAT COLITIS

[0001] CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This application is a continuation of application PCT/EP99/07800 filed October 6, 1999, designating the United States of America, which itself claims priority from European Patent Application EP 98203529.7, filed on October 20, 1998.

[0003] TECHNICAL FIELD

[0004] The invention relates generally to medicine, and particularly to an administration strategy for delivering cytokines or cytokine antagonists at the intestinal mucosa. Preferably, the cytokines or cytokine antagonists are acid sensitive anti-inflammatory agents, such as IL10 and/or soluble TNF receptor. These antagonists may be delivered via an oral route. Preferably, inoculation occurs along with a suspension of recombinant *Lactococcus lactis* cells that are engineered to produce the respective proteins.

[0005] BACKGROUND

[0006] The mammalian immune system is diverse and complex, and includes natural and adaptive immune mechanisms and reactions. The immune system is often described in terms of either "humoral" or "cellular immune" responses. Humoral immunity refers broadly to antibody production and actions by B-cells, while cellular immunity is mediated by cells including T-cells, dendritic cells, neutrophils, monocytes and macrophages. T-cells and B-cells are two categories of lymphocytes.

[0007] One of the mechanisms by which the immune system normally acts and regulates itself includes the production of so-called "cytokines". Cytokines mediate several positive and negative immune responses. Cytokines normally act by binding to a receptor on a target cell. The activity of cytokines can be interfered with in several ways, for example by administration of soluble receptors (extracellular domains of the receptor) or by cytokine analogues or derivatives.

[0008] IL-10 is a cytokine capable of mediating a number of actions and/or effects. It is known that IL-10 is involved in controlling the immune responses of different classes or subsets of Th cells (T-helper cells).

[0009] Inflammatory bowel disease ("IBD") refers to a group of gastrointestinal disorders characterized by a chronic nonspecific inflammation of portions of the gastrointestinal tract. Ulcerative colitis ("UC") and Crohn's Disease ("CD") are the most prominent examples of IBD in humans. They are associated with many symptoms and complications, including growth retardation in children, rectal prolapse, blood in stools (*e.g.*, melena and/or hematochezia), wasting, iron deficiency, and anemia, for example, iron deficiency anemia and anemia of chronic disease or of chronic inflammation. The etiology or etiologies of IBD are unclear. Reference hereto is made in Wyngaarden and Smith (eds.) *Cecil's Textbook of Medicine* (W.B. Saunders Co. 1985), Berkow (ed.) *The Merck Manual of Diagnosis and Therapy* (Merck Sharp & Dohme Research Laboratories, 1982), and *Harrison's Principles of Internal Medicine*, 12th Ed., McGraw-Hill, Inc. (1991).

[0010] The incidence of IBD varies greatly with geographic location. A collaborative study was commenced in Europe. It illustrated an incidence of 10.4 per 100,000 for UC and of 5.6 per 100,000 for CD, with 40% and 80% respectively higher incidences for UC and CD in northern centres when compared to those in the south. As both UC and CD are long time afflictions, they correspond to real disturbances in the quality of life. Crohn's disease has a bimodal age distribution of onset, showing striking peaks in incidence at 20 and at 50 years of age. A higher incidence and more grievous disease profile is associated with those that peak at a younger age. This makes CD especially poignant as afflicted adolescents and young adults are virtually deprived of the high expectations of life particularly associated with this age group.

[0011] Ulcerative colitis refers to a chronic, nonspecific, inflammatory, and ulcerative disease having manifestations primarily in the colonic mucosa. It is frequently characterized by bloody diarrhea, abdominal cramps, blood and mucus in the stools, malaise, fever, anemia, anorexia, weight loss, leukocytosis, hypoalbuminemia, and an elevated erythrocyte sedimentation rate ("ESR"). Complications can include hemorrhage, toxic colitis, toxic megacolon, occasional rectovaginal fistulas, and an increased risk for the development of colon cancer.

[0012] Ulcerative colitis is also associated with noncolon complications, such as arthritis, ankylosing spondylitis, sacroileitis, posterior uveitis, erythema nodosum, pyoderma gangrenosum, and episcleritis. Treatment varies considerably with the severity and duration

of the disease. For instance, fluid therapy to prevent dehydration and electrolyte imbalance is frequently indicated in a severe attack. Additionally, special dietary measures are sometimes useful. Medications include various corticosteroids, sulphasalazine and some of its derivatives, and possibly immunosuppressive drugs.

[0013] Crohn's Disease shares many features in common with ulcerative colitis. Crohn's Disease is distinguishable in that lesions tend to be sharply demarcated from adjacent normal bowel, in contrast to the lesions of ulcerative colitis which are fairly diffuse. Crohn's Disease predominately afflicts the ileum (ileitis) and the ileum and colon (ileocolitis). In some cases, the colon alone is diseased (granulomatous colitis) and sometimes the entire small bowel is involved (jejunoileitis). In rare cases, the stomach, duodenum, or esophagus are involved. Lesions include a sarcoid-type epithelioid granuloma in roughly half of the clinical cases. Lesions of Crohn's Disease can be transmural including deep ulceration, edema, and fibrosis, which can lead to obstruction and fistula formation as well as abscess formation. This contrasts with ulcerative colitis which usually yields much shallower lesions, although occasionally the complications of fibrosis, obstruction, fistula formation, and abscesses are seen in ulcerative colitis as well.

[0014] Treatment is similar for both diseases and includes steroids, sulphasalazine and its derivatives, and immunosuppressive drugs such as cyclosporin A, mercaptopurine and azathioprine. More recently developed treatments, some still in clinical trials, involve systemic administration (by injection) of TNF blocking compounds such as TNF-antibodies or soluble TNF receptor.

[0015] IBD represents a genuine problem in public health because of the absence of etiologic treatment. Although many patients are managed successfully with conventional medical therapy, such as anti-inflammatory corticosteroid treatment, most will have recurrent activity of disease, and two-thirds will require surgery.

[0016] The cause of inflammatory bowel diseases is unknown. The pathogenesis of CD and UC probably involves interaction between genetic and environmental factors, such as bacterial agents, although no definite etiological agent has been identified so far. The main theory is that abnormal immune response, possibly driven by intestinal microflora, occurs in IBD. It is well established ~~is~~ that T-cells play an important role in the pathogenesis. Activated T-cells can produce both anti-inflammatory and pro-inflammatory cytokines. With

this knowledge in hand, IBD can be counteracted in a rational manner. Novel anti-inflammatory therapies, which make use of ~~neutralising~~ neutralizing monoclonal antibodies or anti-inflammatory cytokines, show the possibility to modulate the immune disregulations causative to IBD. A highly prominent and effective new therapy is systemic treatment with anti-TNF monoclonal antibodies as mentioned above. Single intravenous doses, ranging from 5 to 20 mg/kg, of the cA2 infliximab antibody resulted in a drastic clinical improvement in active Crohn's disease. The use of systemically administered recombinant IL-10 in a 7 day by day treatment regime using doses ranging from 0.5 to 25 μg/kg showed reduced Crohn's disease activity scores and increased remission. A number of very promising therapies, either tangling pro-inflammatory cytokines or the establishment of ~~T-cell~~ T-cell infiltrates, are currently emerging from experimental models. All these strategies however require systemic administration. The severe complications of IBD can be seriously debilitating, and eventually may lead to death.

[0017] In U.S. Patent 5,368,854, assigned to Schering Corp., a method is disclosed for using IL-10 to treat inflammatory bowel diseases in mammals. In this method, the cytokine is administered to a mammal having IBD. The administration of IL-10 as described in this reference is parenteral, such as intravascular, preferably intravenous. Such a route of administration for a (human) patient suffering from IBD is, however, not without drawbacks. A much easier and more convenient way would be oral administration of a medicament comprising a cytokine such as IL-10 or a cytokine-antagonist which has a similar therapeutic activity. More importantly, localized release of the therapeutic agent allows for higher efficacy and less unwanted side effects due to systemic activities.

[0018] In WO 97/14806, assigned to Cambridge University Technical Services Ltd., a method is disclosed for delivering biologically active polypeptides and/or antigens by using non-invasive bacteria, such as *Lactococcus*, by intranasally administering the polypeptides to the body, especially at the mucosa.

[0019] However, treating an inflammatory bowel disease such as chronic colitis or Crohn's disease with an acid sensitive cytokine like IL-10, is a very delicate and difficult task to accomplish. Therefore, a system needs to be developed wherein the active compound (*e.g.*, a cytokine or a soluble receptor) is delivered directly at the place where the compound is expected to exert its activity taking into account the acid sensitivity of many cytokines,

particularly IL-10, since, after oral administration, the delivery vehicle needs to pass through the acidic environment of the stomach. Furthermore, various digestive enzymes degrade polypeptides as they pass through the stomach and the gut. Last, but not least, *in situ* administration of the agent may allow one to reach therapeutically effective concentrations difficult to achieve by most systemic routes of administration due to systemic toxicity or other limitations.

[0020] SUMMARY OF THE INVENTION

[0021] The invention generally relates to an administration strategy for delivering cytokines, preferably of acid sensitive anti-inflammatory agents, such as IL10 and/or a soluble TNF receptor, via the oral route to the intestinal mucosa. The invention preferably involves inoculation along with a suspension of live recombinant *Lactococcus lactis* cells engineered to produce the respective proteins. For example, mice having a chronic inflammation of the distal colon induced by administration with dextran sulfate sodium (DSS). The treatment, as scored by histological evaluation, clearly showed a regression of the inflammation and disease symptoms. The finding is highly unexpected since, in order to exert activity at the colon following oral administration, the delivery system had to pass the acidic environment of the stomach and the upper part of the small intestine.

[0022] BRIEF DESCRIPTION OF THE DRAWINGS

[0023] FIG. 1 depicts the schematic maps of the plasmids used. P1 is the lactococcal P1 promoter as in Waterfield et al, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococcal Usp45 (van Asseldonck et al, 1990), mil 10 is a DNA fragment encoding the mature part of murine interleukin 10, tr55 is a DNA fragment encoding the soluble part of type 1 TNF receptor, H6 is a fragment encoding 6 histidine residues, Em^r is the erythromycin selection marker. The DNA sequences of pTREX1 (SEQ ID NO:5), pT1NX (SEQ ID NO:6), pTIMIL10 (SEQ ID NO:7), and pT1TR5AH (SEQ ID NO:8) are listed in the enclosed sequence listing and incorporated herein by reference.

[0024] FIG. 2 illustrates the protein profile following SDS-PAGE of the culture supernatant of the indicated strains after immunoblot, revealed with anti-murine interleukin 10 (panel A) or anti-murine type 1 TNF receptor and anti-6 His (panel B) antisera.

[0025] FIG. 3 is a bar graph depicting the average of colon length of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pTREX1~~ MG1363(pTREX1) was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1TR5AH~~ MG1363(pT1TR5AH) was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis strain* ~~MG1363pT1MIL10~~ MG1363(pT1MIL10)was orally administered.

[0026] FIG. 4 is a bar graph depicting the average of epithelial damage score in the distal colon of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pTREX1~~ MG1363(pTREX1) was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1TR5AH~~ MG1363(pT1TR5AH) was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was orally administered.

[0027] FIG. 5 is a bar graph depicting the average of inflammatory infiltrate score in the distal colon of groups of mice in which: a) chronic colitis had been induced with DSS, b) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pTREX1~~ MG1363(pTREX1) was orally administered, c) chronic colitis had been induced with DSS and to which subsequently *L. lactis strain* ~~MG1363pT1TR5AH~~ MG1363(pT1TR5AH) was orally administered and d) chronic colitis had been induced with DSS and to which subsequently *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was orally administered.

[0028] FIG. 6 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates normal tissue in untreated animals.

[0029] FIG. 7 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates animals pretreated with DSS to acquire chronic colitis.

[0030] FIG. 8 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates animals pretreated with

DSS to acquire chronic colitis to which *L. lactis* strain ~~MG1363pT1MIL10~~ MG1363(pT1MIL10) was subsequently orally administered.

[0031] FIG. 9 shows representative sections of mice distal colon stained with haematoxylin and eosin. Specifically, the picture shown illustrates animals pretreated with DSS to acquire chronic colitis to which *L. lactis* strain ~~MG1363pTREX1~~ MG1363(pTREX1) was subsequently orally administered.

[0032] FIG. 10 is a graph illustrating statistical evaluation of the histology. The colon sections were randomly numbered and interpreted blind. Scores from individual mice were subsequently decoded and the regrouped numbers were analyzed statistically. The DSS colitis panel shows histological sum scores for the distal colon of blank mice and of mice induced with DSS to acquire chronic colitis, either untreated or treated with *L. lactis* cultures. The score is a sum of scores for epithelial damage and lymphoid infiltrate, both ranging between 0 and 4. Groups of mice (n = 10) were alternatively treated with MG1363, MG1363(pTREX1) or MG1363(pT1MIL10) (= IL-10) for two (= 2w) or four (= 4w) weeks. Some of the cultures were irradiated with uv (= + uv) prior to inoculation, which reduced cell viability over 10^6 times. The IL-10-/- colitis panel shows histological sum scores of groups (n = 5) of 7 week old untreated, TREX treated and IL-10 treated female 129 Sv/Ev IL-10-/- mice. The histological score is a sum of the degree of inflammation in the proximal, middle and distal colon, all ranging between 0 and 4. Error bars represent s.e.m.

[0033] FIG. 11 is a graph that shows the representation of bacterial viability after irradiation as measured at OD_{600}.

[0034] DETAILED DESCRIPTION OF THE INVENTION

[0035] In order to achieve the recovery of a patient suffering from an IBD, it is necessary to restore the damaged cells and the organ comprising the damaged cells, for instance the colon. The solution to the above described technical problem is achieved by providing the embodiments characterized below.

[0036] According to the invention, cytokine-producing Gram-positive bacterial strain or a cytokine antagonist producing Gram-positive bacterial strain is used for the preparation of a medicament to treat inflammatory bowel disease.

[0037] The cytokine or cytokine antagonist to be produced by the bacterial host strain is, for instance, IL-10, a soluble TNF receptor or a cytokine analogue such as the IL-12 derived p40 homodimer (an IL-12 antagonist), an Interferon-γ-antagonist, an IL-1 antagonist or a virus-coded cytokine analogue such as EBV BCRF1 (Baer et al., 1984), whereas the Gram-positive bacterial strain preferably is a *Lactococcus* species, and more preferably, a *Lactococcus lactis*.

[0038] Other Gram-positive bacterial strains to be used for the purpose of the current invention are *Bacillus subtilis*, *Streptococcus gordonii, Staphylococcus xylosus*, or a *Lactobacillus* species, such as *L. bulgaricus*, *L. salivarius, L. caseï, L. helveticus, L. delbrueckii* or *L. plantarum*.

[0039] The inflammatory bowel diseases such as a chronic colitis, Crohn's disease and ulcerative colitis can be treated according to the invention with an appropriate dosage of the active cytokine compound, preferably IL-10 or soluble TNF receptor. The treatment unexpectedly restores the diseased colon to an apparently normal and healthy state.

[0040] IL-10 can be administered alone or in combination with at least one additional therapeutic agent. Examples of such additional therapeutic agents include corticosteroids, sulphasalazine, derivatives of sulphasalazine, immunosuppressive drugs such as cyclosporin A, mercaptopurine, azathioprine, and another cytokine. The co-administration can be sequential or simultaneous. Co-administration generally means that the multiple (two or more) therapeutics are present in the recipient during a specified time interval. Typically, if a second agent is administered within the half-life of the first agent, the two agents are considered co-administered.

[0041] The invention disclosed herein thus concerns a localized delivery of IL-10 through *in situ* synthesis by recombinant *L. lactis*. As a result thereof, the inflammation is reduced by 50% in chronic colitis induced with DSS, and prevents the onset of colitis in IL-10 -/- 129 Sv/Ev mice. So the method is equally efficient in comparison to powerful, well-established and accepted therapies relying on the systemic administration of anti-inflammatory proteins.

[0042] The vector, *L. lactis*, is a Gram positive food grade organism which is believed to be totally harmless. It is a non-colonizing micro-organism. Accurate dosage and timing during treatment, shown here to be of great importance, can thus easily be obtained.

[0043] The critical requirement for viability of the vector is shown in the current invention. This indicates the need for *in situ* synthesis of IL-10. The vector is indeed capable of achieving this by showing *de novo* synthesis of IL-10 in the colon.

[0044] An efficient novel concept for protein-based treatment in the intestinal tract is herein disclosed. The treatment can be given by the oral route, which is by far the most desirable for pharmacological formulations. It can exert effects up to the distal colon using a compound with intrinsic sensitivity for the route used. This method bypasses the need for systemic administration. It opens the possibility for the localized delivery of substances, which are unstable or difficult to produce in high quantities. Intrinsically, it is very cost effective. In comparison to systemic delivery, the method may provide for sustained and localized presence of IL-10 at concentrations higher than desirable or even achievable with systemic delivery, especially with regard to latent side effects.

[0045] Some terms used in the current description are, for sake of clarity, explained hereafter.

[0046] Generally, the term "symptoms" refers to any subjective evidence of disease or of a patient's condition. This includes evidence as perceived by the patient. Examples of symptoms of IBD include diarrhea, abdominal pain, fever, melena, hematochezia, and weight loss.

[0047] The term "signs" refers generally to any objective evidence of a disease or condition, usually as perceived by an examining physician or features which would reveal themselves on a laboratory evaluation or other tests such as an ultrasonic study or a radiographic test. Some examples of signs of IBD include abdominal mass, glossitis, aphtous ulcer, anal fissure, perianal fistula, anemia, malabsorption, and iron deficiency. Occasionally, signs and symptoms overlap. For example, the patient complains of blood stools (a symptom), and a laboratory test of a stool sample is positive for blood (a sign).

[0048] The phrase "appropriate dosage" or "effective amount" means an amount or dosage sufficient to ameliorate a symptom or sign of an autoimmune condition or of an undesirable or inappropriate inflammatory or immune response. An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the method, route and dose of administration and the severity of the side affects.

[0049] With "cytokine" is meant a polypeptide factor produced transiently by a range of cell types, acting usually locally, and activating the expression of specific genes by binding to cell surface receptors.

[0050] With "antagonist" is meant a compound that binds to but does not activate receptors, and hence inhibits the action of an agonist competitively.

[0051] "Agonists" are compounds that bind to and activate receptors (e.g., endogenous ligands such as hormones and neurotransmitters, chemically synthesized compounds, natural products like alkaloids).

[0052] The invention is further explained by the following methods used in the current invention.

[0053] Culture media

[0054] GM17 is M17 (Difco, St. Louis, MO, US) supplemented with 0.5 w/v % of glucose. GM17E is GM17 supplemented with 5 μg/ml of erythromycin. BM9 contains per liter 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of NaCl, 2 mmol of $MgSO_4$, 25 mmol of $NaHCO_3$, 25 mmol of Na_2CO_3, 0.1 mmol of $CaCl_2$, 5 g of glucose and 5 g of casitone (Difco). BM9E is BM9 supplemented with 5 μg/ml of erythromycin.

[0055] Recombinant DNA techniques.

[0056] PCR amplification of DNA was performed with VENT polymerase and using conditions recommended by the manufacturer. DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out essentially as described (Sambrook et al., 1990). *L. lactis* was transformed by electroporation of cells grown in the presence of glycine (Wells et al., 1993).

[0057] The plasmid pT1MIL10 (FIG. 1) was constructed by subcloning a PCR fragment, obtained with the primers (CAGTACAGCCGGGAAGACAAT (SEQ ID NO:1) and GCACTAGTTAGCTTTTCATTTTGAT (SEQ ID NO:2)) and performed on a cDNA clone containing mIL10 coding sequence. For the design of this strategy, we made use of the mIL10 cDNA sequence as given in EMBL acc. nr. M37897. By utilizing the above-mentioned primers, the mIL10 fragment could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX (FIG. 1), which is a pTREX1 derivative (Wells and Schofield in: Lactic Acid Bacteria: current advances in metabolism,

genetics and applications. F. Bozoglu & R. Bibek, Eds., *Nato ASI Series H*, Vol.98, p. 37. Springer-Verlag, 1996.)

[0058] The plasmid pT1TR5AH (FIG. 1) was constructed by subcloning a PCR fragment, obtained with the primers (CTGGTCCCTTCTCTTGGTGAC (SEQ ID NO:3) and CCACTAGTCTATTAATGATGATGATGATGATGCGCAGTACCTGAGTCCTGGGG (SEQ ID NO:4)) and performed on a cDNA clone containing sTNFr55 coding sequence. In designing this strategy, we made use of the TNFr55 cDNA sequence as given in EMBL acc. nr. L26349. By utilizing the above-mentioned primers, the sTNFr 55 fragment was provided with a 6his tag at the 3'end and could be subcloned as a blunt – SpeI fragment, after treatment with kinase and SpeI, in the NaeI-SpeI opened plasmid pT1NX.

[0059] Both plasmids code, downstream from the lactococcal P1 promoter, for fusion genes between the secretion leader from Usp45 (Van Asseldonk et al., *Gene*, 95, 155-160,1990) and mIL10 and sTNFr 55, respectively. Upon secretion, the leader sequence is cleaved off.

[0060] Identification of recombinant proteins

[0061] Recombinant mIL10 and msTNFr 55 could be observed in the supernatant of cultures of ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) and ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH), respectively (FIG. 2). For this test, 5 ml aliquots of the cultures were extracted with 2 ml phenol and the proteins were subsequently prepared from the organic phase by precipitation with 10 ml of ethanol. A part of the precipitate, equivalent to 1 ml of culture supernatant, was subjected to SDS-15% PAGE and immunoblotting. Culture samples were taken at relevant times in the growth phase of the bacteria, as described below.

[0062] The culture supernatant of ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) contained, on average, 1 μg/ml of murine IL10. Murine IL-10 activity of the supernatant was measured using a murine mast cell line MC/9 (Thompson-Snipes, L. et al., *J. Exp. Med.* 173, 507, 1991). Human IL-10 binds to murine IL-10R as was demonstrated by transfection experiments (Ho, A.S.Y et al., *PNAS* 90, 11267, 1993; Liu, Y. et al., *J.Immunol.* 152, 1821, 1994). 1 U/ml of IL-10 is defined as the amount of IL-10 that is able to inhibit 50% the level of IFN-gamma production of conA activated splenocytes (Fiorentino, D.F. et al., *J.Exp.Med.* 170, 2081, 1989). The ED50 for this effect is typically 0.3-0.6 ng/ml. When measured along with a standard of known activity (Biosource International, CA) the ~~MG1363[pT1MIL10]~~

MG1363(pT1MIL10) culture supernatant revealed an activity of approximately 8000 U/ml. Berg et al. (*J. Clin. Invest* 98, 1010-1020) report a specific activity of approximately 1.0×10^7 U/mg for recombinant mIL10. From these considerations, and taking into account the variations in the method used, we concluded that the recombinant mIL10, present in the ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) culture supernatant, displayed full biological activity. No IL10 activity could be detected in the supernatant of the control cultures, MG1363 or ~~MG1363[pTREX1]~~ MG1363(pTREX1).

[0063] The culture supernatant of strain ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH) contained, on average, 200 ng/ml msTNFr 55. Loetscher et al. (1991) showed that complete inhibition of TNF cytotoxic activity by sTNFr 55 was only obtained from a molar ratio of 1000 : 1 of sTNFr 55 to TNF and higher. The soluble recombinant TNFr 55 which had been recovered from the culture supernatant of MG1363(pT1TR5AH) showed an equal inhibitory effect on TNF as had been reported for the indigenous product. This was demonstrated by mixing up and thus competing out a titration series of TNF with a titration series of recombinant sTNFr and measuring TNF activity in a cytotoxicity assay as described (Espevik, T and Nissen-Meyer, 1986).

[0064] Pretreatment of the mice

[0065] For the induction of chronic colitis, mice were pretreated as described by Kojouharoff et al. *Clin Exp Immunol* 107, 353, 1997. Six to eight weeks old female Balb/c mice received four cycles of treatment with DSS. Each cycle consisted of 5% DSS in the drinking water for 7 days, followed by a 10-day interval during which they received normal drinking water. Four to six weeks after completion of the last DSS cycle, mice were treated with the *L. lactis* strains as indicated.

[0066] The invention is further explained by the use of the following illustrative examples.

[0067] **Examples**

[0068] **Example 1**

[0069] **Treatment of the mice with live *L. lactis***

[0070] Storage of expression strains.

[0071] Freshly streaked cultures of the *L. lactis* expression strains were inoculated in 10 ml of GM17 or GM17E depending on the absence or presence of an expression plasmid and grown overnight at 30°C. The overnight cultures were diluted 1/100 in fresh GM17 or GM17E and pregrown for 3 hours at 30°C. The cells were harvested by centrifugation and resuspended in BGM9 or BGM9E, depending on the presence of plasmids. These cultures were grown for 5 hours at 30°C. The protein profile of these cultures was analyzed by performing Western immunoblotting on an equivalent of 1 ml of culture supernatant using either antiserum directed towards sTNFr 55 or IL10 respectively. The protein profile of sTNFr 55 and IL10 is shown in the appropriate lanes (FIG. 2). 5 ml of the original GM17 or GM17E overnight cultures were supplemented with 5 ml of glycerol and stored at –20°C. These stocks were used as starter material for several experiments. Protein analysis throughout a series of individual experiments showed that a high degree of reproducibility in the production of the recombinant proteins could be obtained by this procedure.

[0072] Weeks 1 and 2

[0073] Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. The cells were harvested by centrifugation and resuspended in 1 ml BM9 or BM9E. Control, healthy mice and mice with induced colitis were inoculated on a daily basis with 100 µl aliquots of these cell suspensions.

[0074] Weeks 3 and 4

[0075] Stock solutions of *L. lactis* strains were diluted 1/200 in 10 ml GM17 or GM17E and grown overnight at 30°C. These cultures were diluted 1/25 in 10 ml of BM9 or BM9E and grown for 3 hours at 30°C. Aliquots of 200 µl were intragastrically (peroral) administered into mice on a daily basis.

[0076] **Example 2**

[0077] Determination of histological score

[0078] Histological score was determined essentially as described by Kojouharoff et al. I 107, 353, 1997.

[0079] Mice were ~~euthanised~~ euthanized by cervical dislocation. The colon was removed and washed with PBS. The distal third of the colon was cut longitudinally, laid on filter paper and fixed with 10% formalin in PBS overnight. Sections of the parafin-embedded material were made longitudinally. Three 3-μm sections were cut at an intermediate distance of 200 μm. The sections were stained with haematoxylin-eosin. Histological analysis was performed in blind fashion. Mice were scored individually, and each score represented the mean of three sections.

[0080] Histology was scored as follows:

[0081] Infiltration: 0, no infiltrate; 1, infiltrate around crypt bases; 2, ~~Infiltrate~~ infiltrate reaching to L. muscularis mucosae; 3, extensive infiltration reaching the L. muscularis mucosae and thickening of the mucosa with abundant oedema; 4, infiltration of the L. submucosa.

[0082] Epithelial damage: 0, normal morphology; 1, loss of goblet cells; 2, loss of goblet cells in large areas; 3, loss of crypts; 4, loss of crypts in large areas and/or foci of polyploid regeneration.

[0083] Colonic length was measured immediately after dissection and placement on a paper towel. The pathology of chronic colitis is, amongst other parameters, ~~characterised~~ characterized by a decrease in length of the colon and by epithelial damage and infiltration of lymphocytes to a more or less substantial extent. FIG. 3 clearly shows an increase in colon length after the treatment of the inflamed mice with ~~MG1363[pT1MIL10]~~ MG1363(pT1MIL10) and, although to a lesser extent, after the treatment of the mice with ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH).

[0084] FIGS. 4 and 5 show the onset of recovery from chronic colitis, in which mice treated with MG1363(pT1MIL10) appear to improve more extensively than those mice which had been treated with ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH).

[0085] FIG. 4 shows the histological score of epithelial damage whereas FIG. 5 shows inflammatory infiltrate, both determined as described previously.

[0086] FIGS. 6-9 shows the histology of normal tissue, compared to inflamed and treated tissue.

[0087] In the normal histology, one can observe a continuous array of crypts of equal length. In the crypts, numerous goblet cells can be observed. A low number of lymphocytes

is present in the mucosa. No lymphocytes are present in the submucosa. In the inflamed tissue, one can see the disappearance of the ~~organised~~ organized crypt structures, ranging from differences in length to complete absence of structure. Also, in the relicts of the crypts no goblet cells are present. One can observe a large increase of the thickness of the mucosa due to a massive infiltration of lymphocytes. The lymphocytes tend to form ulcerations. In severe cases, infiltration of lymphocytes can also be observed in the submucosa. The epithelium, however, remains intact. The negative control of treatment with MG1363(pTREX1) shows a pathology reminescent of that of heavily inflamed tissue. Mice treated with MG1363 (pT1MIL10) show an almost complete restitution of the normal histology, revealing only slight remainders of infiltrating lymphocytes in the mucosa. Mice treated with ~~MG1363[pT1TR5AH]~~ MG1363(pT1TR5AH) show an intermediate degree in pathology.

[0088] FIG. 10 shows the statistic evaluation of histological scores obtained from individual mice following treatment with the indicated *L. lactis* strains (group size = 10). The score was recorded after blind interpretation of slides from the distal colon as described (Kojouharoff et al., 1997). Each mouse was interpreted according to 3 longitudinal slides, equally spaced over the circumference of the colon. Both lymphoid infiltrate and epithelial damage were rated from 0 to 4 points and values for both parameters were summed for every mouse. Normal blank mice showed a histological score of 1 point. The mice induced for colitis are slightly over 5 points. All of the control groups for *L. lactis* treatment fluctuate around this number, with possibly a slightly higher tendency in some groups. The mice treated for 14 days with mIL-10 producing *L. lactis*, followed by 14 days of recovery however show an average of approximately 3 points. This is a decrease of nearly 50% in the pathology when measured against the difference between untreated and blank control groups. The reduction is significant ($p = 0.0151$).

[0089] **Example 3**

[0090] Due to the culture conditions used, a minor amount (40 ng) of mIL-10 is present in the supernatant of the inoculation suspension. To investigate whether this IL-10 brings about the observed reduction in the histological score we included treatment with UV killed IL-10 producer strains. These cultures were UV irradiated immediately prior to the inoculation. FIG. 11 shows that irradiation reduced the bacterial viability to less than 1 in 10^6 cfu so that no further accumulation of IL-10 was observed. This was not associated with cell

lysis since no drop in OD_{600} was observed and no IL-10 precursor could be detected in the culture supernatant. The irradiation does not affect IL-10 bioactivity. Diseased mice treated for 2 or 4 weeks with the UV dispatched cultures show no difference in colon histology when compared to any of the control groups positive for enterocolitis. The fate of the residual IL-10 in the inoculation medium is most likely denaturation and breakdown in the stomach and duodenum. The acidity of the stomach, prior at pH 1.5, rises to pH6 immediately after inoculation. After 5 minutes a pH of 4 is reached, which further drops from 3.5 to 2.5 in the interval between 30 and 60 minutes after inoculation. IL-10 detected in the stomach 5 minutes after inoculation rapidly decreases in concentration and was only found in trace amounts in the duodenum at 30 minutes after inoculation. At later time-points, no IL-10 was detected here nor in the jejunum or ileum.

[0091] **Example 4**

[0092] Seven serial inoculations of 3.4×10^9 cfu of MG1363(pT1MIL10) were given to 129 Sv/Ev IL-10-/- mice, thereby respecting 1 hour intervals. The intestine was prepared out 30 minutes after the last inoculation and divided in the morphologic compartments. Immediately the tissues were ~~homogenised~~ homogenized in PBS with 1% BSA and 0.05% NaN_3. Cfu of MG1363(pT1MIL10) were determined as 7×10^6 in the stomach, 2.6×10^8 in the duodenum, 2.8×10^7 in the jejunum, 4×10^8 in the ileum, 8.4×10^8 in the caecum and 7×10^8 in the colon. We have detected 70 ng of soluble IL-10 in the colon homogenate. None of the upstream compartments showed any IL-10 content. From this it is concluded that recombinant *L. lactis* can actively produce IL-10 in the colon.

[0093] **Example 5**

[0094] **Prevention of enterocolitis in IL10-/- mice**

[0095] The capacity of the approach described above was tested to prevent the onset of colitis in 129 Sv/Ev IL10-/- mice. These mice spontaneously developed a generalized enterocolitis in the frame between three and eight weeks of age (Kuhn et al., *Cell*,1993;75:263-274). Inflammatory changes first appear in the cecum, ascending and transverse colon of 3-wk-old mutants. Progressive disease in aging IL10-/- mice was characterized by an increased number of multifocal inflammatory cell infiltrates composed of mononuclear cells and neutrophils accompanied by moderate epithelial hyperplasia and slight mucin depletion from goblet cells. Small epithelial erosions and crypt abscesses were

occasionally present and inflammation rarely involved the submucosa. IL10-/- mice used in our studies showed a less severe inflammation as described due to "clean" rather than "conventional" conditions of our animal facility.

[0096] When these mice are treated from week 3 on, for 6 to 8 weeks with either anti IFN-γ or anti-IL-12 colitis can be prevented (Rennick et al., *J-Leukoc-Biol.*,1997 Apr; 61(4):389-396). We treated 3 weeks old mice by daily intra-gastric inoculation with IL-10 producing *L. lactis*. The mice were treated for 4 weeks with either mid-log or end-log cultures whilst an untreated group was kept under identical conditions. FIG. 10 shows histological scores obtained as described (Berg et al., *J-Clin-Invest*;1996, Aug 15;98(4):1010-1020), with the exception that we did not examine the caecum. The nontreated mice show a mean histological score of approximately 4.5 points. This fits well with reported data, provided one takes into account the contribution of the caecal scores in these values and the slight age difference. The group of mice treated with MG1363(pT1MIL10) shows a mean histological score of 1.5 points which is only slightly over values reported for 3 week old mice (Berg et al., *J-Clin-Invest*;1996, Aug 15;98(4):1010-1020). As it is the sum of 3 values ranging from 0 to 4 points, this is considered as a very low score. From these data it is clear that the development of colitis can be prevented by this treatment.

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ABSTRACT

An administration strategy for the delivery at the intestinal mucosa of cytokines or cytokine antagonists, preferably of acid sensitive anti-inflammatory agents, such as IL10 and/or soluble TNF receptor via the oral route. Preferably, inoculation occurs along with a suspension of recombinant *Lactococcus lactis* cells, which had been engineered to produce the respective proteins.

SEQUENCE LISTING

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Remaut, Erik

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<120> USE OF A CYTOKINE-PRODUCING LACTOCOCCUS STRAIN TO TREAT COLITIS

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APPENDIX D

(Article, *Science* 2000, p. 1352-55)

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idence for such stabilization has been seen in solution studies, and direct MHC class II-CD4 binding was not detected (*31*). These results and the data presented here instead suggest that the function of CD4 may be to "boost" or "trigger" the early phase of activation. Once that process is under way, CD4 seems to be excluded from the central core of the synapse, perhaps owing to the formation of some lattice-like structure by the remaining molecules.

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16. GFP fusion constructs were transfected into D10 by electroporation as described (*32*). After 1 to 2 weeks, these cultures were expanded into 24-well dishes, screened by flow cytometry, and sorted for GFP expression. Established clones were subjected to phenotypic analysis by staining for CD4, CD3ε, TCR, CD28, and CD25 as well as proliferation analysis against CA 134–147 and the variant peptides E8T and I5G.
17. Supplemental Web material is available at *Science* Online at www.sciencemag.org/feature/data/1050535.shl.
18. Clones were maintained by weekly restimulations as described (*15*), except that cultures were typically not supplemented with exogenous interleukin-2. Activation experiments were performed from 5 to 10 days after the stimulation culture. CH27 is a B cell lymphoma that expresses $I-A^k$, B7-1, and B7-2. Experiments were done at 37°C as described (*14*).
19. GFP intensity data were corrected both for background intensity and for photobleaching. Background level was measured by imaging a dish in the absence of cells. This background level was subtracted from intensity data to obtain background-subtracted data sets. For bleaching, a nonreactive cell was analyzed for surface intensity over time, and the intensity over time was fitted to an exponential decay curve ($I_t = I_0 10^{kt}$, where k is the decay constant, t is the number of times the fluorophore was illuminated at a constant rate, and I_t and I_0 are the initial intensities and intensities at time t). The decay constant k was used to calculate a correction factor ($1/10^{kt}$) for each time point in the experimental data sets. Typical decay constants were −0.01, giving half-lives of about 30 illuminations. Typical experiments involved about 60 to 80 illuminations.
20. Intensities from 340- and 380-nm excitations of Fura-2 were used to make a ratio image. Background calcium levels were obtained from at least five frames before activation and were similar to nonreactive cells from other portions of the field. Increases of 30% above background were found to be well above random fluctuations, and the onset of agonist-driven reactions typically was characterized by at least a 100% increase in a single 15-s time period. Individual cells were analyzed for maximal pixel intensities along the leading edge of cells by a line-scan function. All collected z planes were analyzed and compared to the average from three intensity line scans (taken at different z planes) around the circumference of the cell of interest. Background- and bleach-corrected data were used for all analyses. Rectangular x-y regions encompassing the estimated interface were determined from the differential interference contrast (DIC) image, and z stacks encompassing these regions were used for the interface projections. Max Intensity Projections were used (Metamorph, Universal Imaging, PA).
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Treatment of Murine Colitis by *Lactococcus lactis* Secreting Interleukin-10

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The cytokine interleukin-10 (IL-10) has shown promise in clinical trials for treatment of inflammatory bowel disease (IBD). Using two mouse models, we show that the therapeutic dose of IL-10 can be reduced by localized delivery of a bacterium genetically engineered to secrete the cytokine. Intragastric administration of IL-10–secreting *Lactococcus lactis* caused a 50% reduction in colitis in mice treated with dextran sulfate sodium and prevented the onset of colitis in IL-$10^{-/-}$ mice. This approach may lead to better methods for cost-effective and long-term management of IBD in humans.

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a significant public health problem in Western societies, affecting 1 in 1000 individuals, yet its etiology remains poorly understood. IBD is characterized clinically by chronic inflammation in the large and/or small intestine, the symptoms of which include diarrhea, abdominal pain, weight loss, and nausea. Death can result, in extreme cases, from malnutrition, dehydration, and anemia. IBD is thought to arise from interacting genetic and environmental factors (*1*) and may involve abnormal T cell responses to commensal microflora (*2–4*). Biologically based therapies such as antibodies to tumor necrosis factor (TNF), which is a strong proinflammatory mediator (*5–7*), and recombinant IL-10 (*8*) can ameliorate the disorder.

Because IL-10 has a central role in down-regulating inflammatory cascades (*9*) and matrix metalloproteinases (*10*), it is a likely candidate for use in therapeutic intervention. In this study we have tested a new method of delivering IL-10: in situ synthesis by genetically engineered bacteria (*Lactococcus lactis*), in two mouse models of the disease, one involving treatment of chronic colitis induced by 5% dextran sulfate sodium (DSS) (*11*) and one involving prevention of colitis that spontaneously develops in IL-$10^{-/-}$ mice (*12*). We show that this approach, which depends on in vivo synthesis of IL-10, requires much lower doses than systemic treatment. Neither mouse model mimics all aspects of human IBD, but such models are essential for development of new therapeutic approaches to IBD (*13–20*).

L. lactis is a nonpathogenic, noninvasive, noncolonizing Gram-positive bacterium, mainly used to produce fermented foods. We previously constructed recombinant *L. lactis* strains for production and in vivo delivery of cytokines

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(*21–23*). We have now engineered an [redacted]*tis* strain (*LL-mIL10*) for secretion of biologically active murine IL-10 (mIL-10) (Fig. 1) (*24*, *25*).

To evaluate the efficacy of the new therapeutic concept, we applied daily intragastric inocula (*24*) of 2×10^7 *LL-mIL10* or control *L. lactis* (*25*) to mice in which chronic colitis had been induced by four cycles of administration of DSS in the drinking water for 7 days, alternating with 10-day periods of recovery (*11*, *26*). Treatment was arbitrarily initiated at day 21 after the fourth DSS administration. The predominant epithelial damage was loss of goblet cells and crypts (Fig. 2A) (*25*). A lymphocytic infiltrate was largely restricted to the thickened mucosa of the middle and distal colon; submucosa was affected in about 10% of mice. Inflammation typically persisted for at least 3 months. The chronic phase of inflammation showed complete regeneration of the intestinal epithelial lining. The range of inflammation is shown in histological images from the distal colon (Fig. 2A) and middle colon (*25*).

Histological scores were devised to allow quantification of histological changes (Fig. 3A). Scores from individual mice ($n = 10$) after treatment with different *L. lactis* strains were recorded after blinded interpretation of sections from the distal colon (*26*). Untreated healthy mice had a histological score of 1, whereas mice with the induced chronic colitis and mock-treated control mice had a score of ~5. Mice treated for 14 days with *LL-mIL10* given by gastric catheter, followed by 14 days of recovery, had an average histological score of ~3. This represents a nearly 50% decrease

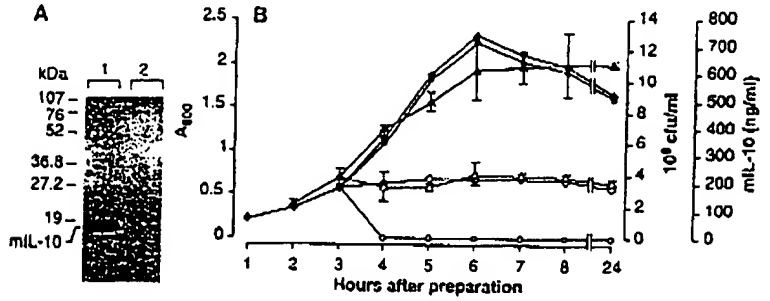

Fig. 1. mIL-10 synthesis by *LL-mIL10*. (A) Western blot analysis of culture supernatant proteins from the mIL-10 producer strain *LL-mIL10* (lane 1) and the vector control *LL-TREX1* (lane 2) (*24*, *25*), revealed with anti–mIL-10 (Pepro Tech EC, London, UK). The position of mIL-10 is indicated. The concentration of mIL-10 in the culture supernatant was 3 μg/ml, as determined by enzyme-linked immunosorbent assay. The biological activity in the culture supernatant was estimated at 10,000 U/ml in a cell proliferation bioassay with the IL-10–dependent mast cell line MC/9 (*33*). When compared with a standard of known activity (BioSource International, Camarillo, California), the recombinant mIL-10 from the *LL-mIL10* culture supernatant revealed full specific biological activity. The NH_2-terminus of this protein was determined, by automated Edman degradation, to be Gln-Tyr-Ser-Arg-Glu, which is identical to that of native mIL-10. (B) A_{600} (◆), colony-forming units (CFU) (●), and mIL-10 concentration (▲) for an *LL-mIL10* culture, prepared as for the inoculation of mice with 2×10^7 bacteria (*24*). Open symbols are for corresponding profiles of an identical culture that was UV-irradiated 3 hours after preparation. UV-irradiation immediately blocked the accumulation of mIL-10 in the culture supernatant and reduced the CFU count by 6 logarithmic units. No lysis was observed (*25*).

0.0151) in pathological symptoms, a more pronounced improvement than that obtained with standard systemic treatment with TNF monoclonal antibodies (mAbs), which leads to a 30 to 40% decrease in inflammation (*26*). In the *LL-mIL10*–treated group, colons of 4 of 10 mice had the histological characteristics of healthy mice, and 4 showed minor patchy remnants of inflammation (*25*); in 2 mice, larger areas remained affected. No further improvement was observed when *LL-mIL10* treatment was extended to 4 weeks; when the daily inoculum was 10^9 bacteria rather than 2×10^7, the improvement in pathology was less pronounced (*27*). Mice with DSS-induced colitis occasionally developed adenomas (*25*), corresponding to development in humans of adenocarcinoma often seen associated with ulcerative colitis. By contrast, no adenomas were seen in mice treated for 14 days with *LL-mIL10*.

We also evaluated the ability of *LL-mIL10* to prevent the onset of colitis in IL-$10^{-/-}$ mice, which spontaneously develop colitis at age 3 to 8 weeks (*12*). This progressive disease is characterized by multifocal inflammatory cell infiltrates (mononuclear cells and neutrophils), moderate epithelial hyperplasia, and slight mucin depletion from goblet cells. Other work has shown that treatment of 3-week-old IL-$10^{-/-}$ mice with recombinant mIL-10, interferon-γ mAbs, or IL-12 mAbs prevents the onset of colitis (*16*, *28*). In our studies, the untreated IL-$10^{-/-}$ mice showed less severe inflammation than that observed by other workers (*16*, *29*). We treated 3-week-old mice ($n = 5$) by daily intragastric inocula of 2×10^7 or 10^9 *LL-mIL10* or control *L. lactis* as for the DSS-induced colitis model (Fig. 2) (*25*). Untreated mice had a mean histological score of ~4.5, whereas the *LL-mIL10*–treated group had a mean score of 1.5, which only slightly exceeds values reported for control mice (*16*) (Fig. 3B). In contrast to results with DSS-induced colitis, treatment effi-

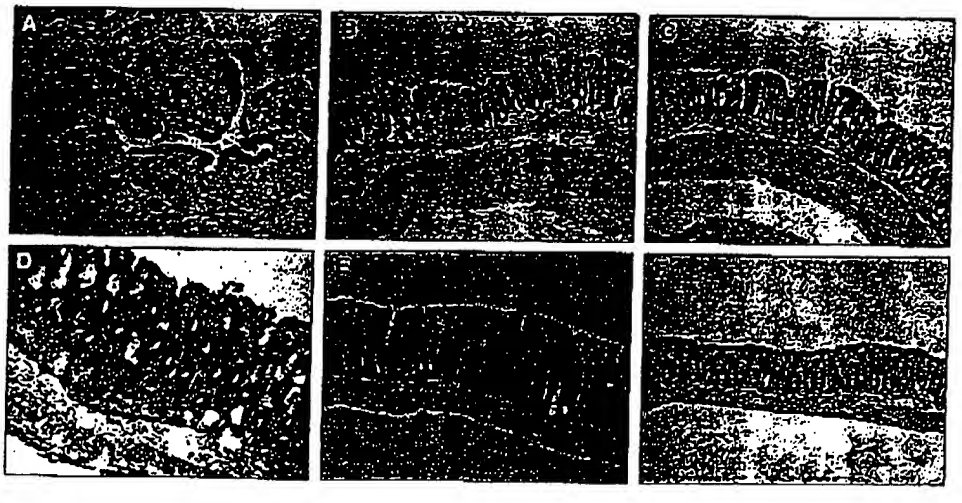

Fig. 2. Intestinal histology of murine colitis models. Images represent sections of the distal colon (magnification, ×100). Hematoxylin and eosin staining. (A to C) Inflammation in the DSS-induced colitis model, established in female Balb/c mice. Lymphocytic infiltrate and disturbance of tissue architecture was observed in untreated mice (A). After treatment by 14 daily intragastric inocula of 2×10^7 *LL-mIL10* per mouse followed by 14 days of recovery, the lymphocytic infiltrate was reduced and the tissue architecture was restored (B). Healthy control mice (C). (D to F) Images from 7-week-old female 129Sv/Ev IL-$10^{-/-}$ mice. Lymphocytic infiltrate is apparent in untreated mice (D) and mice treated for 4 weeks by daily intragastric inocula of 2×10^7 *LL-TREX1* (E) but not in mice treated for 4 weeks by daily intragastric inocula of 2×10^7 *LL-mIL10* (F).

cacy was the same for both inocula concentrations (*27*).

We confirmed that the therapeutic effect was due to mIL-10 synthesized de novo by *LL-mIL10* rather than to residual amounts of mIL-10 in the inocula. Indeed, because of the culture conditions used, a minor amount of mIL-10 (40 ng) (Fig. 1B) was present in the supernatant of the inoculum. The fate of this residual mIL-10 is likely acid denaturation, followed by breakdown in the stomach and duodenum (*25*). Diseased mice (DSS-induced colitis) treated for 2 or 4 weeks with ultraviolet (UV)-killed *LL-mIL10* cultures (Fig. 1B) (*25*) showed no difference in colon histology compared with control mice positive for colitis (Fig. 3A). This result indicates that the therapeutic effects require physiologically active *LL-mIL10*.

We also investigated the synthesis of recombinant mIL-10 by *LL-mIL10* in the intestine of IL-10$^{-/-}$ mice, which cannot themselves synthesize mIL-10. After administration of a total of 2.4×10^{10} *LL-mIL10* (as serial inocula), we detected 7×10^{8} *LL-mIL10* and 7 ng of mIL-10 in the colon (*25*). Hence, these bacteria can actively produce mIL-10 in the colon, albeit at a lower yield than that observed in culture. This result agrees well with recent findings that *L. lactis* is metabolically active in all compartments of the intestinal tract (*30*). Although *LL-mIL10* organisms were present in other areas of the gastrointestinal tract (cecum, ileum, jejunum, and stomach), mIL-10 was not detectable there. Perhaps mIL-10 reached detectable levels only in the colon because in this part of the intestine the protein is not degraded, and the contents move slowly enough to allow its accumulation.

We compared the performance of *LL-mIL10*–mediated mIL-10 delivery with that of standard anti-inflammatory methods: systemic treatment (five daily intraperitoneal injections) with recombinant mIL-10, IL-12 mAbs (*31*), or dexamethasone. All therapies decreased inflammation in DSS-induced colitis by ~50% (Fig. 3A). Our method, however, required a much lower amount of mIL-10. We estimated that 14 daily inoculations of 2×10^{7} *LL-mIL10* delivered ~1 U of mIL-10 per mouse (*25*), i.e., an amount that is several orders of magnitudes lower than the optimized total amount of intraperitoneally injected mIL-10 (1.25×10^{4} U per mouse).

We propose two possible routes by which mIL-10 might reach its therapeutic target. The lactococci may produce mIL-10 in the lumen, and the protein may diffuse to responsive cells in the epithelium or the lamina propria. Alternatively, the lactococci may be taken up by M cells (bacterial size and shape would allow this), and the major part of the effect may be due to recombinant mIL-10 production in situ in intestinal lymphoid tissue. Both routes may involve paracellular transport mechanisms that are enhanced in inflammation. After transport, mIL-10 may directly down-regulate inflammation. Alternatively, autocrine mIL-10 secretion by lymphoid cells, as shown by transfer of Tr1 cells (*32*), epithelial cells, or both, may be induced and may enhance repair.

In summary, the method described here—cost-effective localized delivery of a therapeutic agent that is actively synthesized in situ by food-grade bacteria—may have potential clinical applications for treatment of IBD, particularly as an alternative to systemic treatment. In principle, the method may also be useful for intestinal delivery of other protein therapeutics that are unstable or difficult to produce in large quantities.

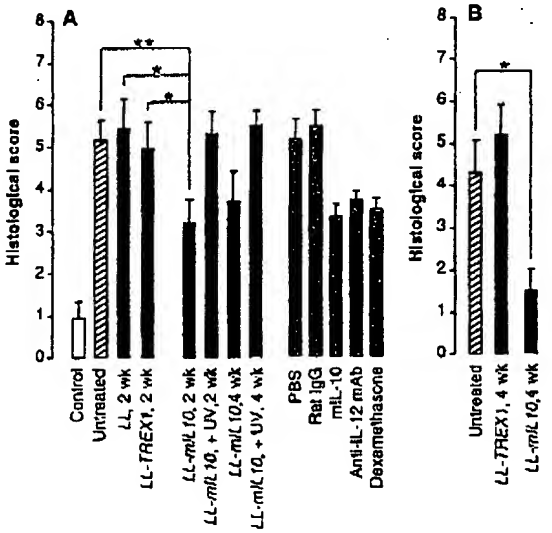

Fig. 3. Statistical evaluation of colon histology. Colon sections were randomly numbered and interpreted in a blinded manner. Scores from individual mice were subsequently decoded, and regrouped numbers were analyzed statistically. Bars represent the mean ± SEM. *$p < 0.025$; **$p = 0.0151$. (**A**) Histological scores (sum of epithelial damage and lymphoid infiltrate, both ranging from 0 to 4) for the distal colon of groups ($n = 10$) of control female Balb/c mice (white bar) and of female Balb/c mice with DSS-induced colitis that were untreated (hatched bar), treated with the indicated *L. lactis* cultures (black bars), or treated with five daily intraperitoneal injections of the compounds indicated (gray bars) (mIL-10: 5 μg per mouse per day; anti–IL-12: 1 mg per mouse per day; dexamethasone: 5 μg per mouse per day; rat IgG: 5 μg per mouse per day). Mice treated daily for 2 or 4 weeks (wk) with 2×10^{7} mIL-10–producing *LL-mIL10* showed significantly reduced inflammation when compared with untreated or control-treated (*LL* or *LL-TREX1*) mice. This effect was not observed when *LL-mIL10* cultures were UV-killed (+UV). (**B**) Histological scores (sum of the degrees of inflammation in the proximal, middle, and distal colon, all ranging between 0 and 4) obtained after blinded interpretation of groups ($n = 5$) of 7-week-old untreated (hatched bar), *LL-TREX1*–treated, or *LL-mIL10*–treated female 129 Sv/Ev IL-10$^{-/-}$ mice (black bars). *LL-mIL10*–treated mice showed significantly less inflammation than untreated mice.

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25. For further details, see *Science* Online (www.sciencemag.org/feature/data/1047997.shl).
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34. We thank I. Bruggeman, H. Devlies, K. Pollinger, and K. Van Laer for technical assistance; J. Wells for pTREX1; T. Velu for mIL-10 cDNA; K. Madsen for 129Sv/Ev IL-10$^{-/-}$ mice; G. Trinchieri for anti–IL-12–producing C17.8 hybridoma cells; J. Van Snick for MC/9 cells; M. Praet for automated tissue processing; and C. Cuvelier, K. Madsen, and P. Vandenabeele for helpful discussion and critically reading the manuscript. LS. is a fellow with the Vlaams Instituut voor de Bevordering van het Wetenschappelijk-technologisch Onderzoek in de Industrie. Supported by grants 1.5567.98N and G005097 of the Fonds voor Wetenschappelijk Onderzoek–Vlaanderen.

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Whistle Matching in Wild Bottlenose Dolphins (*Tursiops truncatus*)

Vincent M. Janik

Dolphin communication is suspected to be complex, on the basis of their call repertoires, cognitive abilities, and ability to modify signals through vocal learning. Because of the difficulties involved in observing and recording individual cetaceans, very little is known about how they use their calls. This report shows that wild, unrestrained bottlenose dolphins use their learned whistles in matching interactions, in which an individual responds to a whistle of a conspecific by emitting the same whistle type. Vocal matching occurred over distances of up to 580 meters and is indicative of animals addressing each other individually.

Bottlenose dolphins show many cognitive and communicative skills that are rare among animals. They are capable not only of generalizing rules, developing abstract concepts and syntactic understanding in an artificial communication system (*1*), but also of vocal learning, i.e., the ability to modify the structure of a vocal signal as a result of experience with those of other individuals (*2*). Although extensive studies in nonhuman primates have not been able to present convincing evidence for vocal learning, this prerequisite for the evolution of spoken language has been demonstrated with much less research effort in bottlenose dolphins (*2*). Dolphins are capable of imitating new sounds accurately at their first attempt, and they keep this ability throughout their life (*3*). Vocal learning is also an important factor in the ontogeny of an individually distinctive signature whistle that each individual develops in the first few months of its life (*4*). Studies on captive individuals have shown that signature whistles are primarily used if animals are out of sight of each other, and they are therefore thought to function in group cohesion and individual recognition (*5*–*7*). However, because bottlenose dolphins are capable of vocal learning, individual signature whistles can be found in the repertoire of more than one individual in captive dolphins (*6*, *8*).

I investigated whether such shared whistles occur in matching whistle interactions between wild dolphins, a phenomenon indicative of their use in addressing specific individuals. Matching interactions were defined as an occurrence in which two whistles of the same type produced by separate individuals occurred within 3 s of each other.

There is often a clear effect of observer presence on dolphin behavior when methods such as tagging or boat pursuits are applied (*6*, *9*). I used a noninvasive passive acoustic localization technique (*10*) to locate calling bottlenose dolphins (*11*). This method uses the differences in the time of arrival of the same sound at different widely spaced hydrophones. Signals from different recording channels were cross-correlated to determine the difference in the time of arrival of a sound at the two corresponding hydrophones. The time-of-arrival comparisons of three pairs of hydrophones then result in three hyperbolas of possible sound source locations. These hyperbolas intersect at the true location of the whistling dolphin. This analysis was conducted with SIGNAL software (Engineering Design, Belmont, Massachusetts). Recordings were conducted in the Kessock Channel of the Moray Firth, Scotland. All data were acquired from the shore, so that no boats or humans were present around the animals.

Vocal interactions between individuals were identified by comparing the distance of the source locations of two successive whistles (minus twice the maximum localization error of 13 m) with the distance that a bottlenose dolphin could travel at its maximum reported swimming speed of 7.5 m/s (*12*) in the interwhistle interval. If the distance between two whistle sources could not have been covered by one individual in the time interval between those whistles, they must have been produced by different individuals.

Five naïve human observers were used to rate the similarity of each whistle interaction using only the extracted contours (*13*) of the whistles; this method is more reliable than computer-based methods that have been used in dolphin whistle studies (*14*). They were allowed to rate whistle similarity on a scale from 1 (=dissimilar) to 5 (=similar). The scores of the different observers were significantly similar (Kappa = 0.34, $z = 16.9$, $P < 0.00001$). Only whistle pairs that reached an average score of more than 3.0 were considered to be matching interactions (*15*).

In a total recording time of 258 min and 43 s from seven different days in July and August 1994 and 1995, a total of 1719 whistles was recorded. These recordings were made with an average of 10 animals present in the channel (quartiles: 7, 10, and 15). Independent counts conducted by a second observer from a higher observation point using binoculars showed that these counts were highly accurate. I could not identify individuals in this study, but a photo-identification study showed that at least 14 different individuals were using this area on a regular basis and that occasionally groups of more than 20 animals were present (*16*). Nine hundred ninety-one of the recorded whistles had a sufficient signal-to-noise ratio on all hydrophones for their source location to be determined. In this sample, 176 whistle interactions were found, of which 39 were classified as matching interactions (Fig. 1). In both matching and nonmatching interactions, 80% of the interwhistle interval was less than 1 s. The mean distance between matching individuals was 179 m (standard error: 22.8 m); the maximum was 579 m. Distances between animals in matching interactions were significantly smaller than those of animals in nonmatching interactions (Kolmogorov-Smirnov Two-Sample Test, two-tailed, $D = 0.291$, $P < 0.025$) (Fig. 2). A randomization test (*17*) showed that this number of matching interactions was signifi-

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